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Infection and Spread of Root Rot Caused by *Heterobasidion parviporum* in *Picea abies* Stands after Thinning: Case Studies on Former Pasture and Meadow Lands

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Abstract: Afforestation of former agricultural lands is a common practice in several countries. This is beneficial for avoiding diseases carry-over from previous forest generations and to expand forest areas. However, several biotic and abiotic risks have been reported in such stands, including a higher risk of *Heterobasidion* root rot after thinning. Therefore, this study investigates the spread of *Heterobasidion* root rot in three *Picea abies* (L.) Karst. plantations in Latvia established on former pasture and meadow lands and subjected to forest management practices. Initially, to determine average infection rate, we sampled all standing trees (157 in total) along transects within sampling areas. On the transects, the proportion of trees suffering from *Heterobasidion* root rot varied from 16 to 33% among stands. Based on those data and observed dieback over all the stands, we established circular sample plots in disease centres where all trees and stumps were analysed. The average infection rate in the circular plots varied from 34 to 41%. Obtained *Heterobasidion* isolates were analysed with species-specific primers and were all determined to be *Heterobasidion parviporum* Niemelä & Korhonen. Isolates were paired to detect the number and size of genotypes. Of 141 genets examined, 99 were isolated from only one tree or stump, while 42 formed genets including two or more trees or stumps indicating spread of infection through root contacts. The total number of *Heterobasidion* genets per hectare in studied stands varied from 72 to 484. The following conclusions were reached: (i) stands on former non-forest lands (pastures and meadows) can be highly susceptible to *Heterobasidion* infection after thinning, (ii) the pathogen may form expanding territorial clones in both former pasture and meadows and (iii) stump treatment with biological or chemical control agents is recommended to prevent *Heterobasidion* infections.

Keywords: Norway spruce; *Heterobasidion* root rot; primary infection; secondary infection; first rotation forest; afforestation

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1. Introduction

Changes in land use from pastures, meadows, or agricultural fields to forest plantations is a common practice in many countries. For instance, in the European Union, 12.9 million hectares of abandoned lands were afforested in 1995–2015 [1]. Agricultural lands differ from lands with previous forest coverage, and thus forest management strategies should consider the most appropriate tree species and potential risks [2–4]. Therefore, several studies have compared stand growth rate and growth conditions in forest lands vs. former agricultural lands [5,6].

One of the most commonly planted conifer tree species in former agricultural lands in Latvia and other Baltic and Nordic countries is Norway spruce (*Picea abies* (L.) Karst.) [7].

Norway spruce is highly susceptible to root rot caused by *Heterobasidion* spp. [8–13]. In Latvia, the country-scale inventory revealed that about 22% of spruce trees contained heart rot, mostly caused by *Heterobasidion parviporum* Niemelä & Korhonen, which results in severe economic losses [14,15]. More than half of the territory of Latvia is covered by forest and the forest sector contributes almost 20% to total exports from Latvia [16]. Consequently, reduction of economic losses caused by root rot is crucial.

Infected previous-generation stumps are important for transfer of *Heterobasidion* infection to subsequent tree generation through root contacts [10,11,17–21]. Therefore, forest planting in infection-free areas and afforestation on former agricultural lands or pastures are important strategies to decrease infection potential [22].

Nevertheless, several studies have reported similar butt rot incidence rate in former fields or grazing land in comparison with former forestlands [23] or even severe damage in former agricultural lands [24–27]. Modelling based on stump infection rate with *Heterobasidion* in spruce stands revealed that former pastures and arable lands are more susceptible to root rot than forest stands [28]. A more recent study form Czech Republic indicated differences in infection rate among different previous land uses, indicating higher infection in former arable lands than in former pastures [29].

Higher infection rate on former agricultural lands has been related to different factors: (i) soil chemical parameters such as high carbonate content, high nitrogen content or overall unbalanced nutrient status [4,30–32], (ii) soil compaction that stimulates the superficial root system of Norway spruce [26], (iii) biotic factors such as lack of fungi antagonistic to *Heterobasidion* [4,26,31,33–36], and (iv) increased percentage of grass cover [30].

Forest management practices, especially cuttings, are significant for the establishment of *Heterobasidion* infections in stands on former agricultural lands where primary spore infection of freshly cut conifer stumps is the main route for disease spread. In contrast, in former forest lands, mycelial infections originating from the old stumps or roots can be a more significant source of disease spread. Therefore, several studies on former agricultural land have focused on root rot development after first thinning [37–39]. Although the incidence of *Heterobasidion* root rot is highly dependent on the degree of stump infection, the possibility of direct root infection cannot be completely omitted. For instance, Rönnberg et al. [40] showed high incidence of *Heterobasidion* root rot in unthinned spruce stands planted on pastureland. Thor and Stenlid [41] found 0.5–8.1% of trees infected in unthinned spruce stands established on former agricultural land and even up to 16.9% infected in an unthinned stand in former pastureland.

Secondary infections occur when hyphae of the pathogens spread to root systems of adjacent trees via root contacts, which can produce expanding disease centres [42,43]. To obtain detailed knowledge on secondary infection, Swedjemark and Stenlid [44] analysed the population structures of *Heterobasidion annosum* s.l. in spruce stands on former arable lands and found that seven years after thinning, 63% of trees have decay symptoms and were infected by mycelial spread from stumps. In another Swedish study, comparing the growth rate of artificially inoculated *Heterobasidion* mycelia in the stem and roots of Norway spruce growing on forest land and arable land, concluded that on average, *Heterobasidion* growth rate in both tree and stump roots did not differ significantly between former arable and forest sites [19].

However, the previously mentioned studies were carried out on former agricultural lands in Sweden and Scotland and the results are not necessarily applicable to other soil and forest types as well as different management practices (both agricultural and forestry). Comparable studies have not been performed in Norway spruce stands in Latvia or neighboring countries, indicating the need for such studies. In a recent Latvian study dealing with *Heterobasidion* root rot in Scots pine stands, Zaļuma et al. [45] reported a lower expansion rate of *Heterobasidion* root rot in pine plantations established on former agricultural land in comparison with former forestland. Consequently, in addition to the previously mentioned studies, it is very important for practical forestry in Latvia to obtain

detailed, more specific information about the spread of *Heterobasidion* root rot in Norway spruce stands on sites with non-forest history.

The aim of this work was to investigate the distribution patterns (primary and secondary infection) of *Heterobasidion* spp. in managed Norway spruce plantations established on former meadows and pasturelands in central part of Latvia.

2. Materials and Methods

2.1. Study Sites and Experimental Design

The study was established in three Norway spruce stands in Central Latvia, in the municipalities of Babite, Auce, and Olaine (Figure 1). In this region, average annual temperature ranges from 6.8 to 7.9 °C and annual precipitation rate is 574 to 636 mm. For Central Latvia, dry pine and spruce forests on sandy or loamy soils, water-saturated areas (peat bogs, wet forest types, flood plain meadows, etc.) with peat soils or drained forests and a large proportion of former agricultural fields on loamy soils are characteristic. The first-rotation spruce plantations in the study represent this variety: a drained forest within a region of peat bogs (Stand 1), a spruce plantation within a region dominated by agricultural fields (Stand 2), and a dry-type (*Oxalidosa*) stand (Stand 3). Characteristics of the investigated areas are presented in Table 1.

Table 1. Description of Norway spruce stands selected for the study: general information, forest characteristics, and details of sampling area and soil parameters.

	Stand 1	**Stand 2**	**Stand 3**
General information			
Location	56°84′ N, 23°77′ E	56°38′ N, 23°12′ E	56°82′ N, 24°07′ E
Municipality	Babite	Auce	Olaine
Altitude, m	17	85	13
Annual rainfall, mm	600–650	550–600	650–700
Forest data			
Total area, ha	8.7	2.75	1.54
Age, years	73	82	52
Average height, m	27.1	28.8	23.5
DBH, cm	35	30	24
Yield per ha	342	387	268
Forest type	*Vacciniosa mel.*	*Myrtillosa mel.*	*Oxalidosa*
Thinning years *	2007, 2015	1991, 2001	1987, 2017
Previous land use	Pastureland	Wet meadow	Meadow
Other tree species (<5% admixture)	*Betula pendula*	*Populus tremula*	*Pinus sylvestris*
Sampling area			
Length of transect, m	500	400	250
Area of transect, m^2	1000	800	500
Total No. of circle sample plots	11	6	5
Total area of circles, m^2	3454	1884	1570
Soil			
pH (KCl)	4.1	5.9	4.6
C, (g/kg)	146	103	106
N, (g/kg)	7.6	8.5	7.5
P, (g/kg)	0.03	0.06	0.03
K, (g/kg)	0.02	0.05	0.02
Ca, (g/kg)	4.4	8.9	6.9
Mg, (g/kg)	0.16	0.88	0.25

* All stands were thinned twice. No data of the exact thinning period and season is available.

All stands had selective or sanitary thinning (Table 1); since after thinning, stumps were not treated with any control agents to prevent infection of root rot, at least potentially, stumps were exposed to primary infection of *Heterobasidion*.

Soil samples for chemical analysis were collected at 0–20 cm depth along transects every 50 m. Chemical analyses of soil were conducted using established standard methods (International Organization for Standardization (ISO) standard) at LSFRI Silava, Laboratory of Forest Environment, Salaspils, Latvia. Samples were prepared for analysis according to ISO 11464:1994. The soil pH was determined in 1 M KCl (soil—extractant mixture 1:2.5). Total carbon content was determined using an elemental analyser ELTRA CS530 (Eltra GmbH, Haan, Germany) according to LVS ISO 10694. The concentration of total N was determined using the Kjeldahl method (ISO 11261:1995) and P was assayed by colourimetry using the ammonium molybdate spectrometric method in 1 M HCl solution (soil/extractant mixture 1:5). Concentration of K, Ca, and Mg was determined by atomic absorption spectrophotometer with an acetylene-air flame in 1 M HCl extract.

Figure 1. Location of the study sites in the central part of Latvia.

To determine average infection rate, we sampled all standing trees within a 2-m-wide transect parallel to the longest edge of each stand with starting points in the middle of the shortest edge. Health status was assigned to each tree in the transect: (i) visually healthy crowns and (ii) trees with severe dieback symptoms, dead and windthrown trees [9]. To detect the pathogen under laboratory conditions, wood samples were collected from all trees on the transect with the aid of an increment borer (one sample per tree at root collar height). Additionally, to characterise stand growth rate, we measured the diameter at breast height and, for living trees, the tree height (Table 1).

Based on the transect data about the distribution of *Heterobasidion* infected trees and visual observation of potential infection centers, we established from five to eleven circular sample plots per stand (10 m radius, 314 m^2) where all trees and stumps were sampled and examined for *Heterobasidion* fruit bodies. In addition, stumps were classified as either stumps from the 1st or 2nd thinning. Broken dead tree trunks of different heights were categorized as stumps. Wood samples from trees were collected with an increment borer (one sample per tree at root collar height) and from stumps—either with the same method or with the aid of an axe if they were heavily decomposed. Wood samples from each tree were placed into individual plastic tubes and stump samples into individual plastic bags. All sampled trees and stumps (both in transects and circular plots) were numbered and their location mapped. Field work was carried out from March 2019 to April 2020.

2.2. Isolation and Identification of Heterobasidion Genotypes

In the laboratory, all wood samples were surface sterilized by flaming and placed on petri dishes containing malt extract agar (MEA) media (for tree samples) or Hagem agar media (for stump samples). After a 5 to 7-day incubation, the plates were examined under a Leica stereomicroscope M205C (20–30× magnification) to detect the presence of

Heterobasidion spp., observing its characteristic asexual sporulation (conidiophores and conidia) on wood samples [10]. If *Heterobasidion* was observed, colonies were sampled with sterilised surgical forceps and placed on new MEA media. Outgrowing *Heterobasidion* mycelia were subcultured to obtain pure fungal cultures.

Isolates originating from the same circular sample plot were subjected to pairwise somatic compatibility tests by confronting their mycelia on Hagem agar media in 9 cm Petri dishes in all combinations. The genets were identified either by recording the line of demarcation in the contact zone of confrontation (demonstrating that the genotypes are different) or by observing free fusion of the mycelia (implying that the genotypes were identical) [46].

The results of this test provided an information about the total number and size of *Heterobasidion* genets: number of genets encompassing only one tree or stump and number of genets encompassing two or more trees and (or) stumps. Based on these data, we estimated the number of trees/stumps infected by *Heterobasidion* genets per hectare and per disease centre. To estimate the area and maximum width of each genet including two or more trees/stumps, we used the map of each site with all sampled trees and stumps mapped. We used species specific primers (*Heterobasidion parviporum* specific primers KJ-F and KJ-R and *Heterobasidion annosum* specific primers MJ-F and MJ-R) to determine the *Heterobasidion* species of each genotype [47]. DNA was extracted from fungal cultures representing each genotype using a CTAB method [48]. Two PCR reactions were run with each sample—one with *H.parviporum* and other with *H.annosum* species specific primers listed above. PCR conditions, were a described by Hantula and Vainio [47]. DNA from *H. parviporum* and *H. annosum* was used as a positive control.

2.3. Data Analysis

A chi-square (χ^2) test of independence from the actual number of observations of infected or decaying trees and stumps was used to compare abundance patterns between sites and sample plots [49].

Using the geospatial tool QGIS 3.2.0-Bonn (bc43194) (https://qgis.org/api/3.2/), tree spatial location was visualised and the width and the area of genets including two or more trees/stumps was measured (tool "MeasureLine" and "MeasureArea"). The maximum expansion rates were calculated as a reference taking years from the first thinning for each stand (2007, 1991 and 1987, respectively, Stand 1 to 3) or from the second thinning (2015, 2010 and 2017, respectively, Stand 1 to 3).

To assess which factors have an impact on the number of trees per genet, we used generalized linear mixed models (GLMM) assuming a Poisson error distribution. The full model included number of trees per genet as a response variable and as fixed effects we tested site, stand age class (older stands (Stand 1 and 2) vs. younger stand (Stand 3), disease centre infection indicators (classes of infection (<40% vs. >40%), stump and dead tree proportion) and as a random factor in all models we included disease centre. Models were calculated using the program package lme4 [50].

Area and width of genets including two or more trees/stumps as well as maximum expansion rates (area and width) were tested for normality using the Shapiro and Wilk normality test at significance level $\alpha = 0.001$ [51]. If normality was assessed, one-way analysis of variance (ANOVA) with Fisher LSD post-hoc test was calculated [52]. If normality was not assessed, nonparametric Wilcoxon test (unpaired samples) was used to compare parameters. For calculations, R version 4.0.2 was used [53].

3. Results

3.1. Incidence of Dieback and Heterobasidion Infection

In total, in all three study sites, 851 wood samples were collected for *Heterobasidion* isolation (397 from living trees, 118 from dead trees and 336 from stumps). The number of sampled trees and stumps are given in Table 2. In all stands, stumps from both 1st and

2nd thinning were present and in addition there were some trees broken close to their base (mainly in Stand 2) (Table 2).

Table 2. Characteristics and dieback rate in Norway spruce stands on former pastures (Stand 1) and meadows (Stands 2 and 3). (T—transect; C—disease centres (circular sample plots).

	Stand 1		Stand 2		Stand 3	
	T	**C**	**T**	**C**	**T**	**C**
Living trees						
Total number *	72	95	25	73	34	153
Number per hectare	720	275	313	387	680	975
Dead trees						
Total number *	8	41	10	37	8	29
Number per hectare	80	119	125	196	160	185
Stumps						
Total number	n.d.	142	n.d.	96	n.d.	98
No. of 1st thinning stumps	n.d.	65	n.d.	45	n.d.	29
No. of 2nd thinning stumps	n.d.	75	n.d.	39	n.d.	69
No. of other stumps **	n.d.	2	n.d.	12	n.d.	-
Number per hectare	n.d.	411	n.d.	510	n.d.	624

* Several trees were present in both transect and disease centre. ** Category "other stumps" includes trees broken at different heights.

In Stand 2 and 3, chi-square test of independence showed that transect and disease center data have similar tendencies regarding the number of living and dead trees ($p > 0.05$). In contrast, chi-square test for Stand 1 data indicated significant differences in living and dead tree abundance in disease centers and the transect ($\chi^2 = 11.65$; df = 1; $n = 216$) (there were less dead trees in the transect ($\chi^2 = 5.67$) than expected).

Additionally, a chi-square test of independence was performed to examine the numbers of living trees and stumps among sites. The relation between these variables was significant, $\chi^2 = 24.17$ (df = 2, $n = 657$), $p < 0.05$. The highest chi-square values were observed for both living trees and stumps in Stand 3: more living trees ($\chi^2 = 7.52$) and less stumps ($\chi^2 = 7.18$) than predicted.

The transects of both Stand 2 and 3 had several openings, which explains the lower number of trees per hectare in comparison with disease centres (Table 2). In Stand 3, these openings were related to stand planting design (there were several gaps left to facilitate thinning). Stand 3 had higher planting density that reflects in the number of trees per hectare (Table 2).

In addition, fruit bodies were recorded as an indicator of disease of trees and stumps. There were more records of fruit bodies in Stand 1 (two dead trees and 10 stumps with fruit bodies) compared to other stands (there were two fruit bodies recorded in Stand 2 and four fruit bodies in Stand 3, found exclusively on stumps in both stands).

After incubation of 851 collected wood samples, we detected pathogen conidiophores with conidia in 302 samples (35%); infection rate varied among stands (30% in Stand 1 and 39% in Stands 2 and 3) (Table 3).

Chi-square test indicated that in transects and disease centres, the incidence of *Heterobasidion* infection was similar among sites ($p > 0.05$). In addition, the infection incidence of both living and dead trees in disease centres have a similar pattern among sites ($p > 0.05$). In total, we confirmed the presence of the pathogen in 31.8 and 48.3% of all living and dead tree samples, respectively. Infection incidences of living trees among sites was 24.5, 38.6 and 35.2% (Stand 1 to 3, respectively) and of dead trees 58.1, 35.7 and 51.5% (Stand 1 to 3, respectively). In total, we confirmed the presence of the pathogen in 34.2% of all stump samples. Infection incidences varied among stump samples at different sites: 26.1, 39.6 and 40.8% (Stand 1 to 3, respectively). However, the differences were not significant.

Table 3. Frequency (% *) of Norway spruce trees or stumps infected by *Heterobasidion parviporum* in managed forests on former pastures and meadows.

	Stand 1	Stand 2	Stand 3
Transect; %	**16.3**	**28.6**	**33.3**
Living trees; %	12.5	25.7	23.8
Dead trees; %	3.8	2.9	9.5
Disease Centres; %	**33.5**	**41.3**	**38.6**
Living trees; %	11.5	15.5	18.9
Dead trees; %	8.6	7.3	5.4
Stumps; %	13.3	18.4	14.3
Total; %	**29.6**	**38.5**	**38.9**

* Percentage expresses the proportion of trees/stumps where *Heterobasidion parviporum* was detected of all trees/stumps sampled.

3.2. Analysis of Heterobasidion Genets

Pure *Heterobasidion* sp. cultures were successfully isolated from 90.5% of a total of 286 wood samples producing *Heterobasidion* conidiophores with conidia collected from disease centers (76.3, 98.8, and 96.3% from Stands 1 to 3, respectively) (Table 3). Identification with species specific primers revealed that all obtained isolates belonged to species *Heterobasidion parviporum*.

In total, 141 *Heterobasidion* genets were detected resulting in 72 to 484 *Heterobasidion* genets per hectare among study sites (Table 4). Less genets per hectare were detected in Stand 1 (2.9 times and 6.7 times less in comparison with Stand 2 and Stand 3, respectively).

Table 4. Distribution pattern and characteristics of *Heterobasidion parviporum* genets in disease centres of managed Norway spruce stands on former pasture and meadows.

	Stand 1	Stand 2	Stand 3
Genets including one tree/stump			
Infection per hectare	38	143	376
• Trees infected	35	64	274
• Stumps infected	3	79	102
Frequency (%)	21%	25%	56%
Genets including two or more trees/stumps			
Number of trees and stumps per genet (min.-max.)	4.8 (2–12)	4.4 (2–11)	2.6 (2–7)
Infection per hectare	170	302	293
• Trees infected	120	185	191
• Stumps infected	50	117	102
Distance among trees and (or) stumps, m			
• Mean ± SE	9.6 ± 1.7	6.9 ±1.0	3.1 ± 0.5
• Min.-max.	1.8–18.0	2.1–14.6	1.0–6.9
Area, m^2			
• Mean ± SE	56.4 ± 19.7	12.4 ± 4.9	5.9 ± 2.9
• Min.-max.	1.2–137.4	0.3–52.2	1.3–17.3
Frequency (%)	79%	75%	44%
Total infection per hectare	208	445	669

The mean number of trees/stumps per genet was 2.8, 2.1 and 1.4 in Stand 1 to 3, respectively. Genets including one tree/stump were the most abundant in all sites: 13, 27, 59 (i.e., 52, 68 and 78% of all genets per stand), in Stand 1 to 3, respectively. Expanded

genets including two or more trees/stumps were less frequent 12, 13 and 17 (Stand 1 to 3 respectively). Chi-square test indicated differences among abundance of genets including one tree/stump and genets including two or more trees/stumps per sites ($\chi^2 = 6.1$, df = 2, $n = 141$; $p > 0.05$).

Frequency of trees or stumps infected by genets including one tree/stump of all infected trees and stumps was 21, 25 and 56% (Stand 1 to 3, respectively). Chi-square test indicated differences among abundance of trees and stumps infected by genets including one tree/stump per sites ($\chi^2 = 26.3$, df = 2, $n = 259$; $p > 0.05$), in particular abundance of trees and stumps of genets including one tree/stump in Stand 1 was lower than predicted ($\chi^2 = 7.4$) and in Stand 3 higher than predicted ($\chi^2 = 8.4$).

In comparison with Stand 1 and 2, Stand 3 had more isolates and consequently more genets (in total and both genets including one tree/stump and genets including two or more trees/stumps). Stand 3 had a lower number of trees per genet and less expanded genets including two or more trees/stumps (lower distance among trees and area) (Table 4). Modeling the number of trees per genet and including disease centres as a random factor revealed that grouping Stand 1 and 2 together (older stands) vs. Stand 3 identified a significant difference ($p < 0.05$).

The maximum number of trees infected by genets including two or more trees/stumps are presented in Table 4. The largest genets included both living and dead trees and stumps. The longest mean distance between trees and (or) stumps with shared genets was about 7–10 m (maximum 15–18 m) (Stands 1 and 2) compared with values of Stand 3 (mean longest distance 3 m; maximum 7 m) (Table 4). Analysis of variance showed significant differences and post-hoc tests indicated that the genets including two or more trees/stumps in Stand 3 were significantly ($p < 0.05$) smaller in size than genets in Stand 1 (3.3 times smaller) and in Stand 2 (2.3 times smaller).

The maximum area of genets including two or more trees/stumps in Stand 1 was 2.6 to 8 times greater than in Stand 2 and 3 (Table 4). The mean area of genets including two or more trees/stumps was also 5 to 9 time larger in Stand 1. Analysis of variance showed significant differences and post-hoc tests indicated that the genets including two or more trees/stumps in Stand 1 had significantly ($p < 0.05$) larger area than both Stand 2 and 3 ($p < 0.05$).

The maximum expansion length per year calculated from the first thinning was 1.5, 0.5 and 0.2 m (Stand 1 to 3, respectively) and maximum expansion area per year: 11.5, 1.9, and 0.5 m^2 (Stand 1 to 3, respectively). Wilcoxon test showed that also mean expansion indicators (distance and area per year) were higher in Stand 1 in comparison with Stand 2 and Stand 3 ($p < 0.05$).

4. Discussion

4.1. Dieback and Observed Heterobasidion Infection of Norway Spruce on Former Pasture and Meadow Lands

Visual observation of stand health status revealed expansive spread of root rot in the studied Norway spruce stands on former non-forest lands (pastures and meadows) in Latvia. The greater total number of trees and stumps per hectare in Stand 3 is mainly related with greater planting density. The number of stumps indicates intensity of thinning and, therefore, potential infection risk of stumps by *Heterobasidion* spores. Greater abundance of stumps might also favor the mycelial spread of *Heterobasidion* since its growth rate in stump roots is significantly faster than in living trees [19,54]. Moreover, in densely planted stands, where the trees or stumps are closer to each other, the possibility of root contacts is higher, thus allowing greater probability of infection transmission [4].

In Stand 2, there were several trees broken at different heights (Table 2), which indirectly indicates reduced tree vitality. In addition, gaps and a low number of trees per transect might indicate not only a reduced number of trees due to the stand age but also poor current and previous pathological conditions within the stand.

Fruit bodies were found in all study sites, which indicates that environmental conditions were suitable for fruiting of *H. parviporum*. Consequently, the risk of primary infection

by *H. parviporum* spores should be taken into account when planning future thinning in these stands or other similar sites.

The detected *H. parviporum* infection rate (30% to 39%) was on average in line with data from other studies from both former agricultural and forest lands. It indicates that even though no inoculum is present in former non-forest lands, infection can establish and expand as successfully as in forest lands or even more successfully. For instance, in a Swedish study carried out on a spruce stand on former agricultural land, seven years after thinning, 63% of the trees had decay symptoms [45]. In Sweden, modelling of spruce stands including different former land use indicated 2–90% of rotted trees in final cuttings [55]. In Denmark, on average, 23% (max. 94%) of Norway spruce individuals growing on former agricultural or grazing fields were found to suffer from butt rot [23].

The success rate of *H. parviporum* isolation from dead spruce individuals was 35.7 to 58.1%. However, since the pathogen was not detected in all dead spruce individuals, it cannot be excluded that either the infection has not yet spread higher in the stem or that causes other than *Heterobasidion* root rot were responsible for a proportion of declining or dead trees. Other fungal agents as *Armillaria* sp. can cause root rot in Norway spruce stands [56]. In addition, spruce dieback not related to root rot has been observed in several regions in Latvia [57].

The frequency of stumps colonized by *H. parviporum* (34.2% on average) was relatively high in spruce stands planted in former pastures and meadows in Latvia. Viable *Heterobasidion* mycelium can survive in stumps for decades [58]. However, with time, it develops more intensively in deeper layers of stumps [59] and isolation can be difficult due to wood decomposition and presence of other stump colonizing fungi. Since the stumps in the studied stands were not recently cut, total stump infection may be even higher. Infected spruce stumps left after thinning transfer root rot to residual trees and serve as a source of infection [21,54]. Therefore, winter thinning and use of biological control agents containing spores of *Phlebiopsis gigantea* or other preventive substances such as urea is recommended to limit the infection of conifer stumps by basidiospores of *Heterobasidion* spp. [43,60–62], especially in stands with no previous rot incidences such as in stands established on former agricultural lands [4,63,64].

4.2. Heterobasidion Genets

All obtained *Heterobasidion* isolates were identified as *Heterobasidion parviporum*, which is the most common *Heterobasidion* species causing damage in spruce stands in Northern Europe and Latvia [15,65]. *H. parviporum* is adapted to spruce as the host species [66,67] and is therefore more often observed in spruce stands, being predominant or exclusive in some cases [9,68–70], and is able to spread more frequently from stumps to neighbouring trees than *H. annosum* [71]. Korhonen et al. [72] reported that *Heterobasidion* infections in spruce stands on former agricultural lands were mainly caused by *H. parviporum* and concluded that in such first rotation spruce stands without any history of pine growth and woody inoculum, spores of *H. parviporum* are the principal infection source. Moreover, Vasiliauskas and Stenlid [18] showed that *H. parviporum* is better adapted to spruce wood, and spreads more extensively in spruce stems compared to *H. annosum*.

In our study, the number of trees/stumps infected by *H. parviporum* per hectare was 208, 445 and 669 (Stand 1 to 3, respectively) (Table 4). In addition, the total number of *Heterobasidion* genets per hectare in the present study was high: 72, 212, and 484 (Stand 1 to 3, respectively). In comparison, in a Finnish study on *Heterobasidion* infections on clear-felled forests on old forest sites, the number of fungal genets per hectare varied from 2 to 158 (mean 28) [73]. The high infection rate and high number of *Heterobasidion* genets in the present study may indicate a higher susceptibility of Norway spruce to *Heterobasidion* root rot on former agricultural lands compared to old forest lands [2].

The number of genets including only one tree or stump was lower in older stands (Stand 1 and 2) than in the youngest one (Stand 3). Time from the first thinning did, however, not differ significantly between the stands (Table 1). However, even in final cuttings on

old forest sites, genets including one tree/stump and therefore primary infection can be dominant [73]. In general, a lower number of trees infected by the same *Heterobasidion* genet and occupying a smaller stand area indicate that these clones are relatively younger than those comprised of more trees and covering a larger area [44]. However, the age of the genets of *Heterobasidion* may affect the pathogenicity of the fungus, with younger genets being more pathogenic [39].

Development of territorial clones following primary infection of *Heterobasidion* takes at least 7–8 years [42,44,74]. In our study, 22 to 48% of detected genets formed expanding territorial clones, which included 44 to 79% of infected trees and (or) stumps. This indicates that infection in all sites is well established and expanding. Due to well-developed clonality, in Stand 1 and 2, it is most probable that infection was initiated due to the first thinning (12, 28 years ago), and not by the more recent ones (4, 18 years ago) (Stand 1 and 2, respectively). In Stand 3, infection history seems to be much more recent than in other stands and probably more expansive after the last thinning (two-years before sampling) than after the first one (32-years before sampling). In addition, greater planting density in Stand 3 could favour development of territorial clones in a shorter period as indicated previously [35,42,44].

Comparing the maximum size of *Heterobasidion* genets with other studies, we found genets of similar size also reported from stands with a much longer infection history. For instance, Vasiliauskas and Stenlid [18] reported territorial clones of *H. annosum* s.l. up to 20 m in diameter in a 60 year-old spruce stand planted in a previously infected pine site, while in the oldest stands of our study (Stand 1 and 2), territorial clones reached up to 14–17 m. In addition, the largest distance between isolates from the same genet in Finnish study [69] in 70 to 100 year-old spruce stands was 13 m, which is less than in our study on former non-forest lands. Previous studies indicate that *H. parviporum* can form large territorial clones. Stenlid [46] studied a 120-year-old and thinned *P. abies* stand and detected *H. parviporum* territorial clones reaching up to 30 m in diameter. Similarly, up to 40 m long territorial clones of *H. parviporum* were observed in an intensively thinned *P. abies* stands in Serbia [74]. In related studies from Finland, the maximal length of territorial clones varied between approximately 10 and 30 m [9,66] or reached up to 55 m [73]. Our study shows that even 12 to 32 years after thinnings in stands on former pastures and meadows, *H. parviporum* can form large territorial clones.

The maximum number of trees per genet in our study was 12 and this is lower in comparison to stands on former forest lands (i.e., a Finnish study [67] reported 16 trees per genet) and also to stands on former pasturelands, where a greater number of trees (up to 25) forming territorial clones have been reported [40]. However, the real number of infected trees and stumps in our study could be greater than detected due to various reasons: (i) stump decomposition and poor isolation success from some samples, (ii) sampling height of trees (root collar) excluding infection in lower zones, (iii) sample number (one sample per tree) not covering all stem inner surface where decay column can be located, and (iv) limited sampling area (territory of circular sample plot). In addition, to estimate the exact borders and size of *Heterobasidion* genets, a thorough analyses of excavated root systems would be required. Consequently, the size of the genets may actually be bigger.

All the largest *H. parviporum* genets of our study contained three to nine dead trees or stumps. Stumps colonized by *Heterobasidion* provide a substrate from which the fungus can spread and cause infection in standing trees [21]. A study by Capretti and Mugnai [26] indicated that dead wood (buried wood in their study) might be an important factor for extensive spread of the pathogen especially in former pasture soil. Recent studies show that root fragments are important for *H. parviporum* infection transfer [75]. Therefore, both abundance of dead wood and roots could be related with the spread of the pathogen in Stand 1 (former pastureland). The average expansion rate of territorial clones of *H. parviporum* on Stand 2 and 3 (former meadows) (0.25 and 0.10 m·year^{-1}) corresponds to results obtained by Bendz-Hellgren et al. [19], where the average *H. annosum* s.l. growth rate on former agricultural lands was 0.25 m·year^{-1} in spruce stump roots and 0.09 m·year^{-1} in

living tree roots. The average *H. parviporum* growth rate in the Stand 1 (former pasture-land) was high, 0.8 m·year^{-1}, in comparison with results on both forest and arable lands reported by Bendz-Hellgren et al. [19]. This might indicate an erroneous assumption of infection year (i.e., infection had occurred before first thinning via wounding). Since no wounds were observed on trunks, current primary infections probably occurred via root injuries. Spores of *Heterobasidion* are present in soil, where they can maintain their vitality at least for a year and infect injured tree roots [32]. Rönnberg et al. [40] reported a high incidence of *Heterobasidion* root rot in unthinned spruce stands planted on pastureland. In addition, Vollbrecht and Agestam [28] and Capretti and Mugnai [26] indicated that former pastures were highly susceptible to root rot. Capretti and Mugnai [26] also concluded that *Heterobasidion* growth rate in dead wood was particularly high in pasture lands. The increased capacity of *H. parviporum* to colonise dead wood and form fruitbodies in Stand 1 of our study is a probable scenario, explaining the greater expansion of genets.

In research sites, stumps were not treated against *Heterobasidion* root rot, which in a short period resulted in severe damage as reported above. Therefore, we suggest that stump treatment should be considered as a general practice on former pastures and meadows to prevent both primary and subsequent infections, as suggested by other authors [41,43,62,64]. This is particularly relevant for private forest owners who are often managing forests on former agricultural lands, but often do not utilise preventive stump treatments.

Further studies should focus on the presence of *Heterobasidion* spores in soils of former agricultural lands in relation to the soil microorganism community and root injuries.

5. Conclusions

This study generated new data on the ecology and patterns of spread of *Heterobasidion parviporum* in Norway spruce stands on former pastures and meadows. From these novel data, we concluded the following: (i) on average, primary infection via spores in such stands is dominant, but secondary infection increases with stand age, (ii) *H. parviporum* can form large (up to 137 m^2) territorial clones in forests on former pasture and meadow lands causing extensive tree dieback and mortality. Therefore, to reduce economic losses in spruce plantations on former pastures or meadows, stump treatment with biological or chemical control agents that prevent *Heterobasidion* infections should be considered in thinning operations. It is important to facilitate the implementation of stump treatment for private forest owners, who in many cases are owners of forests on former agricultural lands.

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